Immunity, Vol. 15, 303–311, August, 2001, Copyright ©2001 by Cell Press

Analysis of Type 2 Immunity In Vivo with a Bicistronic IL-4 Reporter

Markus Mohrs,[1] Kanade Shinkai,[1] Katja Mohrs,[1] and Richard M. Locksley[1,2]

[1] Howard Hughes Medical Institute
Departments of Medicine and
Microbiology/Immunology
University of California, San Francisco
San Francisco, California 94143

Summary

Effector T cells mediate adaptive immunity and immunopathology, but methods for tracking such cells in vivo are limited. We engineered knockin mice expressing IL-4 linked via a viral IRES element with enhanced green fluorescent protein (EGFP). Reporter T cells primed under Th2 conditions showed sensitive and faithful EGFP expression and maintained endogenous IL-4. After *Nippostrongylus* infection, reporter expression demonstrated the evolution of type 2 immunity from tissue lymphocytes and thence to lymph node $CD4^+$ T cells, which subsequently migrated into tissue. The appearance of $EGFP^+$ $CD4^+$ T cells in tissue, but not in lymph nodes, was Stat6-dependent. Transferred $EGFP^+$ $CD4^+$ T cells from infected animals conferred protection against *Nippostrongylus* to immunodeficient mice. These mice will provide a valuable reagent for assessing immunity in vivo.

Introduction

Host immunity and immunopathology are mediated largely by cytokines, short-lived effector molecules rapidly secreted by different cell types in many different tissue compartments. Analysis of cytokine activities in vivo has been extrapolated from in vitro studies using isolated cells and through generation of cytokine knockout and transgenic overexpressing mice. Although many insights have been gained by these approaches, disadvantages are also apparent. First, analysis of cells in vitro removes them from their in situ environment and from potentially critical signals present in tissues. Second, exogenous cytokines are often provided in amounts that might not be physiologic. Third, knockout or reporter knockin mice preclude the ability to judge the contributions of the cytokine that has been deleted since it can no longer contribute to the immune response. Finally, overexpression and transgenic reporter systems frequently result in the loss of key regulatory elements that may be required to modulate expression when the cytokine is in the genome in its appropriate DNA context.

The need for cytokine reporters that can be introduced without deleting the cytokine itself led us to explore viral internal ribosomal entry site (IRES) elements as a mechanism for faithfully marking cells transcribing cytokines. This approach was applied successfully in mice to map projections of olfactory sensory neurons using an IRES-β-galactosidase reporter (Mombaerts et al. 1996, Wang et al., 1998). Using enhanced green fluorescent protein (EGFP), we elected to mark the endogenous IL-4 gene in mice in an attempt to visualize the host immune response in vivo. Produced by lymphocytes and nonlymphoid cells, IL-4 remains the canonical marker for Th2 cells, which have been implicated in mucosal immunity against parasites and in allergic and atopic responses, including asthma (Finkelman et al., 1997; Wills-Karp, 1999). The ability of IL-4 itself to direct the differentiation of IL-4-producing $CD4^+$ and $CD8^+$ T cells has suggested an autocrine positive-feedback mechanism for the generation of a type 2 immune response in vivo. However, the initial source of IL-4 necessary for Th2 development remains elusive; $CD4^+$ T cells themselves are one candidate population. As such, a functional endogenous IL-4 gene might be critical in attempting to assess the biology in the intact animal. We characterize the generation of mice containing an endogenous reporter for IL-4-expressing cells using both in vitro assays and in vivo infection with *Nippostrongylus brasiliensis* (Urban et al., 1998). The ability to identify IL-4-producing cells in vivo without the need for restimulation or cloning should provide a powerful reagent for assessing protective and pathologic correlates of immunity.

Results

Generation of 4get Mice

The targeting vector consisted of an IL-4 genomic EcoRI fragment comprising exons 3 and 4 with approximately 5 kb of 3′ untranslated sequence (Figure 1A). A loxP-flanked *neomycin* selection cassette linked to the IRES-EGFP construct with the polyadenylation signal from bovine growth hormone was introduced just downstream of the translational stop and upstream of the endogenous polyadenylation signal in the 3′ untranslated region of exon 4. A herpes simplex thymidine kinase expression cassette was cloned to one flank of the targeting construct for use as a counterselectable marker for transgene integration. The construct was electroporated into 129/SvJ-derived PrmCre embryonic stem (ES) cells which express the Cre recombinase under control of the germline-specific protamine promoter, thus allowing Cre-mediated deletion of the *neomycin* gene from the male germline (O'Gorman et al., 1997). G418- and gancyclovir-resistant ES cell clones were screened for correct integration by Southern blot, and targeted clones were injected into C57BL/6 blastocysts. Chimeric males were bred to wild-type BALB/c mice, and offspring were screened by Southern blot for the mutated allele and for deletion of the *neomycin* selection cassette. Heterozygous animals were bred to BALB/c mice, and offspring were screened for the presence of the reporter and absence of the Cre transgene. These mice were interbred to obtain homozygous animals (Figure 1B). The targeted mice, which were healthy and exhibited no obvious phenotype, were designated IL-4/GFP-enhanced transcript, or 4get, mice.

[2] Correspondence: locksley@medicine.ucsf.edu

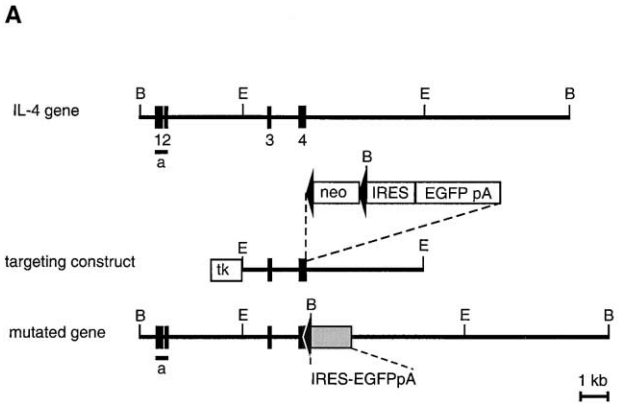

Figure 1. Targeting of the IRES-EGFP Reporter into the Mouse IL-4 Locus

(A) Map of the mouse IL-4 locus, the reporter-targeting construct, and the mutated gene. A genomic 5 kb EcoRI fragment from the wild-type locus (top panel) was mutated by the introduction of a loxP-flanked (filled triangles) drug-selectable *neomycin* cassette (*neo*), followed by an internal ribosomal entry site (IRES) element, enhanced green fluorescent protein (EGFP), and a polyadenylation signal (pA) (middle panel). After successful targeting in PrmCre embryonic stem cells, the *neo* cassette was deleted by Cre-mediated recombination in the male germline of chimeric males, thereby creating the final arrangement depicted at the bottom of the panel. Numbered, filled boxes indicate exons. The following abbreviations are used: tk, thymidine kinase cassette; B, BamHI; E, EcoRI.

(B) Southern blot analysis of DNA from tail biopsies from offspring of heterozygous breeding. DNA was digested with BamHI and hybridized with probe a (dark line, top panel, Figure 1A). A new BamHI site introduced by the targeting construct results in a 7 kb fragment in place of the 17 kb BamHI fragment in the wild-type locus. Genotypes are indicated as wild-type (+/+), heterozygous knockin (+/4get) and homozygous knockin (4get/4get).

Faithful IL-4 Production by 4get Naive CD4$^+$ T Cells In Vitro

Naive (small, resting, EGFP^{neg}, CD62L^{hi}) CD4$^+$ and CD8$^+$ T cells were sorted from 4get mice to >99% purity. Naive T cells were stimulated with anti-TCR/anti-CD28 antibodies and irradiated APC under neutral (IL-2), Th1 (IL-2 with IL-12 plus anti-IL-4), or Th2 (IL-2 with IL-4 plus anti-IFN-γ) conditions and analyzed for EGFP expression at designated times (Figures 2A and 2B). Under Th1 or neutral conditions, no stable EGFP expression was observed (Figure 2D; Grogan et al., 2001). In contrast, under Th2 conditions, EGFP could be detected as early as 36 hr, prior to the onset of cell division (40–45 hr; our unpublished data), consistent with previous reports using sensitive methods for IL-4 detection (Richter et al., 1999; Laouar and Crispe, 2000; Grogan et al., 2001). The proportion of CD4$^+$ T cells expressing EGFP peaked at days 4–5 in vitro, with 72%–92% of cells positive in various experiments (Figure 2A; our unpublished data). Expression of EGFP at the peak of the response on day 3.5 after activation was significantly attenuated in the absence of Stat6 (Figure 2C).

To analyze EGFP expression in relation to cell division, we labeled the membranes of naive CD4$^+$ T cells with the red fluor PKH26 prior to stimulation and polarization (Figure 2D). Although cells stimulated under Th1 or neutral conditions proceeded through cell division and diluted PKH26 intensity, EGFP was not induced. Under Th2 conditions, however, essentially all cells that underwent cell division expressed EGFP and were CD62L^{lo}. The EGFP$^-$ cells remaining in the culture consisted primarily of a population of undivided cells that maintained their CD62L^{hi} phenotype, possibly indicating incomplete activation (Figure 2D; our unpublished data). In the absence of activation using TCR/CD28, EGFP induction and dilution of PKH26 intensity was minimal over 4 days. Of note, the EGFP mean fluorescence intensity (MFI) was not related to the numbers of cell divisions, consistent with the early induction and constant level of IL-4 transcripts as assessed by sensitive RT-PCR methods (Grogan et al., 2001). By comparison, standard methods for intracellular IL-4 trapping after PMA/ionomycin and brefeldin A treatment of 5-day, Th2-primed naive CD4$^+$ T cells revealed 15%–28% IL-4-positive cells (our unpublished data), consistent with published studies (Openshaw et al., 1995; Noben-Trauth et al., 2000; Ouyang et al., 2000).

Polarization of CD4$^+$ T cells from heterozygous 4get mice resulted in the same percentages of EGFP$^+$ cells, but the MFI was only half that from homozygous 4get cells (Figure 2A; Table 1). Naive 4get CD8$^+$ T cells displayed essentially the same activation kinetics under Th2 priming conditions, but the proportion of EGFP-expressing cells and the MFI per cell were less as compared to those of CD4$^+$ T cells (Figure 2B). Of note, the proportional numbers and the MFI of EGFP$^+$ CD8$^+$ T cells were the same using cells from either heterozygous or homozygous 4get mice, consistent with monoallelic IL-4 expression.

To test the fidelity of the endogenous IL-4 genes, CD8-depleted spleen cells from wild-type and 4get heterozygous and homozygous mice were primed with TCR/CD28 antibodies under Th1, neutral, or Th2 conditions for 5 days, restimulated for 48 hr, and the supernatants were analyzed for cytokines by ELISA (Figure 3A). In multiple experiments, cells from these different mice produced comparable amounts of IL-4 and IFN-γ under the various priming conditions, indicating that the endogenous IL-4 gene remained intact and functional in 4get mice. Similarly, when assessed for numbers of IL-4-producing cells with ELISPOT assays, CD4$^+$ T cells from wild-type and 4get mice that were primed under Th2 conditions generated comparable numbers of IL-4-producing cells (Figure 3B), while priming Stat6-deficient 4get cells diminished the numbers of IL-4-producing cells, in accordance with the flow-cytometric analysis and published observations (Figure 2C; Kaplan et al., 1996).

Identification of IL-4-Producing Cells In Vivo

Infection of mice with the helminth, *N. brasiliensis*, induces strong type 2 immunity. Subcutaneously inoculated larvae invade venules, embolize to the lungs after

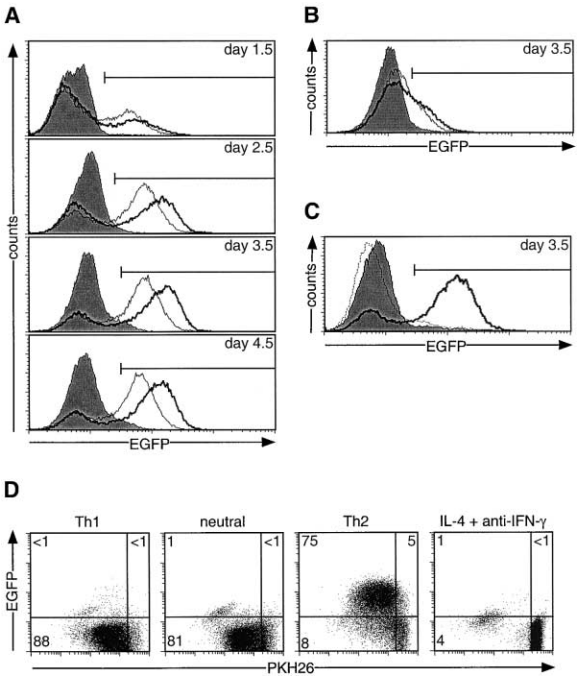

Figure 2. EGFP Expression of 4get T Cells In Vitro

(A) FACS histograms of wild-type (gray fill), heterozygous 4get (thin line), and homozygous 4get (bold line) CD4$^+$ T cells. Naive CD4$^+$ EGFP$^-$ T cells were purified by cell sorting and cultured under Th2 conditions in the presence of antigen-presenting cells. After days indicated, EGFP expression was analyzed after gating on CD4$^+$ cells.

(B) As in (A), but naive CD8$^+$ T cells were purified and primed instead of CD4$^+$ T cells.

(C) As in (A), but Stat6$^{-/-}$/4get (dotted line) CD4$^+$ T cells were compared to wild-type (gray fill) and homozygous 4get cells (bold line).

(D) Purified, naive CD4$^+$ EGFP$^-$ homozygous 4get T cells were labeled with the red fluor PKH26. Cells were cultured under Th1, neutral, or Th2 conditions in the presence of antigen-presenting cells and analyzed on day 4 after gating on CD4$^+$ cells. Cells cultured for 4 days under Th2 conditions but without anti-TCR/CD28 stimulation (far-right panel) demonstrate that dilution of PKH26 intensity requires TCR stimulation.

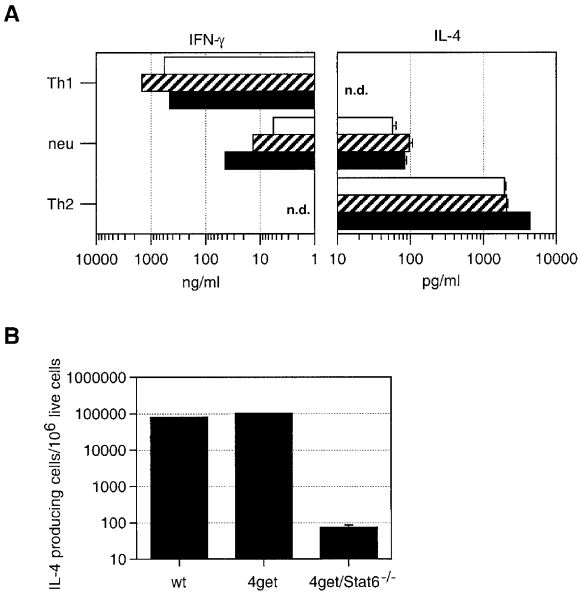

Figure 3. Cytokine Secretion by 4get T Cells

(A) Splenocytes from wild-type (+/+, open bars) and heterozygous (+/4get, hatched bars) or homozygous (4get/4get, dark bars) 4get mice were depleted of CD8$^+$ cells by complement lysis and stimulated under Th1, neutral, or Th2 conditions for 5 days. Cells were washed and restimulated, and supernatants were collected after 48 hr and analyzed for IL-4 and IFN-γ by ELISA. Mean and positive standard deviations of triplicate cultures are shown.

(B) Purified, naive CD4$^+$ EGFP$^-$ T cells from wild-type, 4get, and 4get/Stat6$^{-/-}$ were cultured under Th2 conditions with antigen-presenting cells. After 5 days, cells were restimulated with plate-bound anti-CD3 mAb for 6 hr, and serial dilutions of viable cells were analyzed by ELISPOT. Mean and positive standard deviations of triplicate cultures are shown.

1–2 days, escape into the alveolae, ascend the trachea, descend the esophagus, and mature into egg-laying adults upon arrival in the small bowel after 5–6 days (Finkelman et al., 1997). Infection is characterized by massive T cell and eosinophil infiltration into the lung during larval migration, along with mesenteric lymphadenopathy and intestinal mast cell hyperplasia, mucus production, and elevated IgE levels as worms mature in the gut. Expulsion of adult worms within 10 days is dependent on the development of Th2 cells (Finkelman et al., 1997).

4get mice were infected with *N. brasiliensis* and examined after 10 days. No adult worms could be detected in the intestine at this time. To assess whether the reporter had been appropriately activated in vivo, cell suspensions were created from the lung and mesenteric lymph nodes, as well as from spleen and peripheral lymph nodes that did not drain the site of inoculation of the parasites. Noninfected littermates were examined concurrently.

Approximately 40% of the total CD4$^+$ T cells in the lungs of infected 4get mice spontaneously expressed EGFP without the need for restimulation (Figure 4A).

Table 1. Expression of EGFP in CD4$^+$ T Cells under Th2 Conditions

	Day 1.5		Day 2.5		Day 3.5		Day 4.5	
Genotype	%	MFI	%	MFI	%	MFI	%	MFI
+/+	<1	–	<1	–	<1	–	<1	–
+/4get	34	48	63	87	70	88	66	75
4get/4get	30	68	57	140	73	157	73	136

Purified naive CD4$^+$ T cells from wild-type (+/+), heterozygous 4get (+/4get), and homozygous 4get (4get/4get) mice were incubated with anti-TCR/CD28 mAbs under Th2 conditions. On indicated days, samples of cells were analyzed for the percentage of cells that expressed EGFP (%) and for the mean fluorescent intensity (MFI). Data are representative of three comparable experiments.

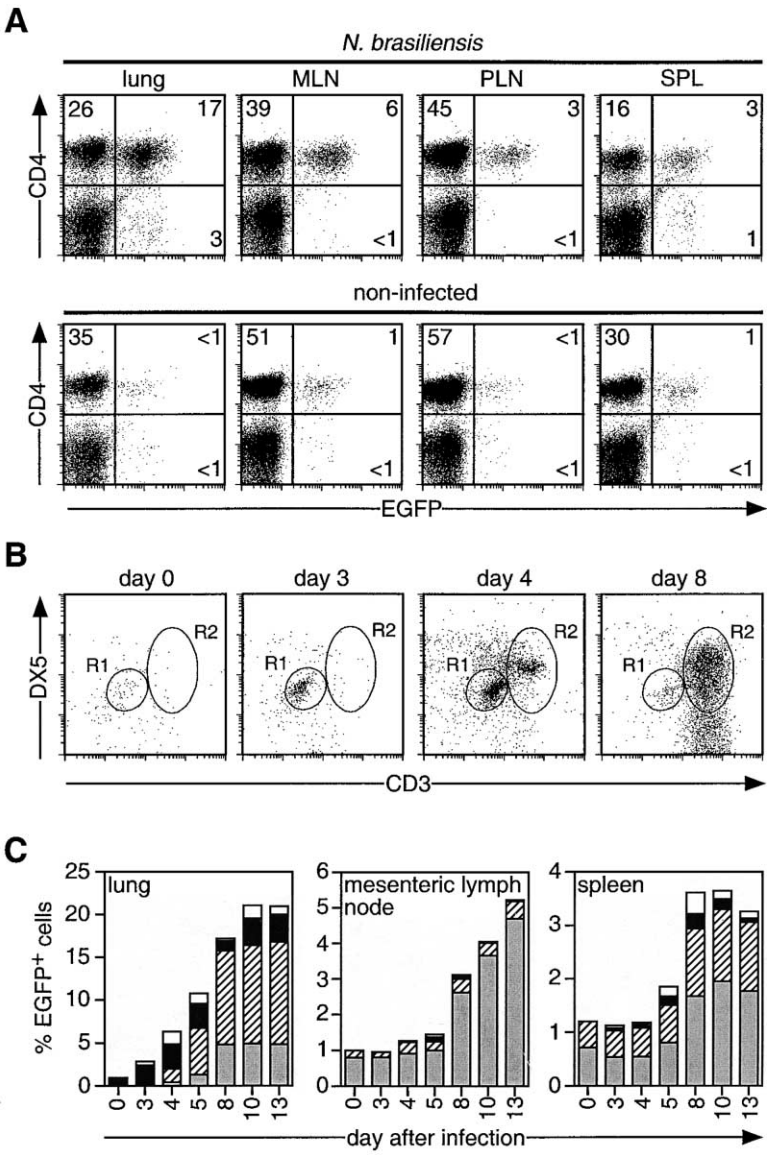

Figure 4. Spontaneous EGFP Expression in 4get Mice Infected with *N. brasiliensis*

(A) Homozygous 4get mice were infected with *N. brasiliensis*. FACS analysis of lung, mesenteric lymph nodes (MLN), peripheral lymph nodes (PLN), and spleen (SPL) was performed after 10 days. Cells from the forward- and side-scatter lymphocyte gate were examined for CD4 and EGFP expression. Noninfected control mice were housed in the same facility as infected mice. Cells from 3 mice were pooled for each analysis. Numbers indicate percentages per quadrant.

(B) As in (A), but the analysis was performed by gating on EGFP$^+$ lung lymphocytes for the expression of DX5 and CD3 on indicated days after infection. R1 = DX5^{lo} CD3^{lo} cells, R2 = DX5$^+$ CD3^{hi} cells.

(C) As in (A), but mice were analyzed on indicated days after infection for the percent of EGFP cells in designated tissues. Dark fill indicates CD4$^+$ CD3^{lo} DX5$^+$ cells, hatched fill indicates CD4$^+$ CD3^{hi} DX5$^+$ cells, gray fill indicates CD4$^+$ CD3^{hi} DX5$^-$ cells, and open fill indicates CD4$^-$ CD3$^-$ DX5$^-$ cells.

Expression in spleen and mesenteric lymph node CD4$^+$ T cells was comparable in various experiments (approximately 15% of the total) and was consistently greater than in nondraining peripheral lymph nodes (approximately 5%). CD8$^+$ T cells, although capable of EGFP expression when primed under Th2 conditions in vitro (Figure 2B), were not activated in vivo (our unpublished data), consistent with experimental evidence that these cells are not required in immunity against *Nippostrongylus* (Brown et al., 1996). Noninfected mice demonstrated baseline EGFP fluorescence in approximately 1% of spleen, lung, mesenteric, or peripheral lymph node CD4$^+$ T cells (Figure 4A). The finding that IgE levels in infected 4get mice were comparable to those in wild-type littermates (1040 $\pm$ 90 ng/ml versus 960 $\pm$ 105 ng/ml, respectively) confirmed that endogenous IL-4/IL-13-mediated Ig class-switching remained functional in these mice.

To examine the kinetics and cell composition of the EGFP-expressing cells, 4get mice were infected with *N. brasiliensis* and examined at various days after infection in conjunction with cell surface markers. After 3 days, when larvae were migrating through the lung, CD4$^+$ T cells in pulmonary, but not in lymphoid tissues, were already expressing EGFP above the background from uninfected mice. The earliest EGFP-expressing lymphocytes were uniformly DX5^{lo} CD3^{lo}, consistent with NK T cells (Bendelac et al., 1997; Figure 4B, R1 gate). These cells reached a peak on day 4 and then abruptly declined on day 8; a small amount of repopulation occurred on days 10 and 13 (Figure 4C). A second population of CD3^{hi} CD4$^+$ T cells appeared in the lung first on day 4 (Figure 4B, R2 gate). Comprised initially of DX5^{hi} cells but then increasingly of DX5^{lo} and DX5$^-$ cells, this CD4$^+$ T cell population eventually dominated the tissue response (Figures 4B and 4C). In the mesenteric lymph nodes, the majority of EGFP$^+$ cells were DX5$^-$ CD4$^+$ T cells, whereas EGFP-expressing DX5$^-$ and DX5$^+$ CD4$^+$ TCR $\alpha\beta^+$ T cells appeared in spleen tissues during this period (Figure 4C). Mediastinal lymph nodes could not

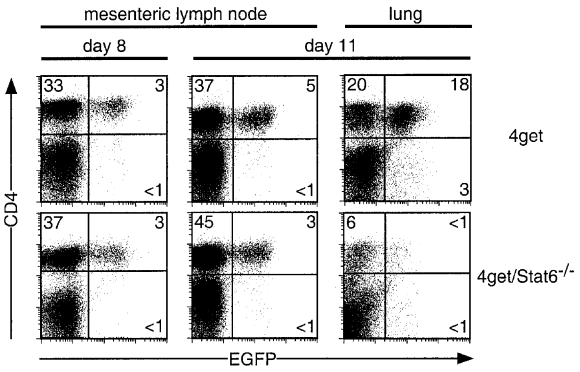

Figure 5. EGFP Expression in 4get/Stat6$^{-/-}$ Mice Infected with *N. brasiliensis*

Homozygous 4get and 4get/Stat6$^{-/-}$ mice were infected with *N. brasiliensis*. FACS analysis of lung and mesenteric lymph nodes was performed on day 8 and day 11 after infection. Cells from the forward- and side-scatter lymphocyte gate were examined for EGFP and CD4 expression. Cells from three mice were pooled prior to analysis. Numbers indicate percentages per quadrant.

be reliably identified early in infection, but by day 13 the EGFP$^+$ cells in these nodes were essentially all DX5$^-$ CD4$^+$ T cells (our unpublished data). Finally, non-T cells, identified as predominantly eosinophils in the lung because of morphologic staining characteristics (our unpublished data), were identified by EGFP fluorescence as a CD4$^-$ CD3$^-$ population that appeared in the lungs beginning on day 4 (Figure 4C).

Activation Requirements for Tissue and Lymph Node Lymphocyte EGFP Expression

Prior studies have implicated signals through IL-4Rα and Stat6 for protective type 2 immunity against *N. brasiliensis* (Urban et al., 1998). Paradoxically, infected Stat6-deficient mice generated normal serum levels of IL-4 and IL-13, despite the finding that IL-4/IL-13 doubly deficient mice were also unable to expel intestinal worms (Finkelman et al., 2000; McKenzie et al., 1999). To investigate these findings further, 4get mice were crossed to Stat6-deficient mice and infected with the parasite. After 11 days, by which time 4get littermates had expelled all intestinal worms, 4get/Stat6-deficient mice had numerous worms remaining in the intestine; this result is consistent with prior studies (Urban et al., 1998; our unpublished data). Unexpectedly, expression of EGFP in CD4$^+$ T cells in the mesenteric lymph nodes was unimpaired at day 8 and only modestly compromised at day 11 by the absence of Stat6 (Figure 5). In contrast, expression of EGFP in CD4$^+$ T cells in the lungs was strikingly impaired both early (day 3) and late (day 11) after infection (Figure 5; our unpublished data). The loss of IL-4-producing tissue lymphocytes was due to a reduction in the numbers of lymphocytes in the lungs (approximately 15% of control) as well as strikingly fewer EGFP$^+$ cells among the infiltrating lymphocytes (less than 10% of control).

CD4$^+$ EGFP$^+$ Effector Cells Mediate Protective Immunity

To confirm that activated, EGFP$^+$ CD4$^+$ T cells remained viable and functional, we used adoptive transfer to immunodeficient recipients to assess whether protective memory could be conferred by effector cells. CD4$^+$ EGFP$^+$ T cells were sorted from the mesenteric lymph nodes of 4get mice infected 12 days previously with *N. brasiliensis*. Additional phenotyping of these cells confirmed that they were uniformly CD62L^{lo} and CD44^{hi}. After they were washed, 7×10^5 cells were used to reconstitute TCR Cα-deficient mice, and after 30 days, recipient mice were infected with *N. brasiliensis*. Prior to infection, the majority (82%) of the transferred CD4$^+$ T cells had become EGFP$^-$ (Figure 6). Ten days after infection, all reconstituted mice had expelled worms from the gut, and the percentage of EGFP$^+$ cells in the mesenteric lymph node CD4$^+$ T cell population had increased to 43%. CD4$^+$ EGFP$^+$ cells were readily detected in both lung and lymphoid tissues. In contrast, nonreconstituted TCR Cα-deficient mice were unable to expel worms. Compared to nonreconstituted infected mice, reconstituted infected mice generated significantly greater levels of IgE in serum ($0.06 + 0.02$ μg/ml versus $205 + 10$ μg/ml, respectively), confirming that B cell isotype switching to IgE could be mediated by EGFP$^+$ 4get T cells. Thus, EGFP$^+$ CD4$^+$ T cells collected during the resolution of acute infection were capable of mediating protective immunity to *Nippostrongylus* in otherwise immunodeficient mice.

Discussion

Cytokines mediate immunoprotective and immunopathologic responses. Despite their obvious importance, it is difficult to track cytokine-producing cells in vivo due to the rapid secretion of cytokines and the short half-lives of the proteins and mRNA transcripts. In an attempt to follow the development of effector immunity in vivo, we have engineered mice containing a bicistronic mRNA linking a readily identifiable reporter, EGFP, to IL-4 gene expression. Cells from these mice revealed that the majority of naive CD4$^+$ T cells express IL-4 upon activation under Th2 priming conditions, a result substantially greater than previously appreciated using standard methods. Indeed, fully 30% of CD4$^+$ T cells activated under Th2 conditions expressed EGFP prior to the onset of cell division. Using a prototypic type 2 immune challenge—infection with the helminth *N. brasiliensis*—we demonstrate several novel and previously unappreciated insights using these reporter mice. First, a CD4$^+$ CD3^{lo} population of IL-4 effector cells appeared initially in lung tissues in response to parasite migration, suggesting that rapid cytokine activation can occur in situ prior to antigen processing and trafficking to the lymph nodes by dendritic cells. Second, Stat6 was not required for the activation of IL-4 expression in lymph node cells but was required for the appearance of IL-4-expressing cells in tissue. Lastly, we confirm that antigen-experienced (memory) helper T cells capable of mediating protective immunity likely derive from effector T cell populations, as demonstrated previously for cytotoxic CD8$^+$ T cells (Jacob and Baltimore, 1999; Opferman et al., 1999). Taken together, these mice will provide valuable insights for understanding the development and maintenance of effector immunity in vivo.

A number of the problems inherent with existing meth-

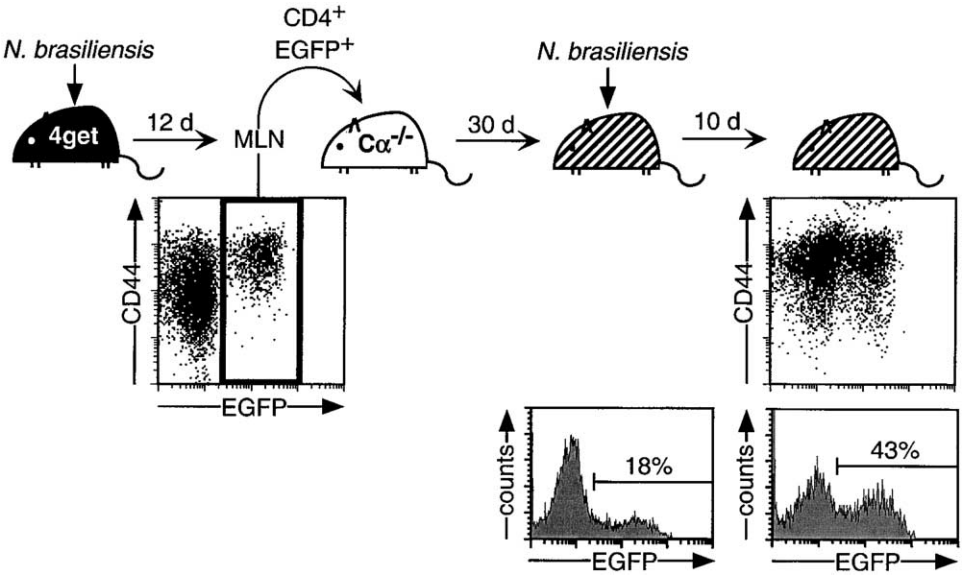

Figure 6. Adoptive Transfer of EGFP$^+$ Effector T Cells into TCR Cα-Deficient Mice

Homozygous 4get mice (depicted in black) were infected with *N. brasiliensis*. After 12 days, mesenteric lymph nodes (MLN) were analyzed for CD44 and EGFP expression in CD4$^+$ T cells, and 7×10^5 purified EGFP$^+$ CD4$^+$ T cells were transferred intravenously into TCR Cα-deficient mice (depicted in white). Thirty days later, MLN of recipient mice (hatched) were analyzed for EGFP expression on gated CD4$^+$ cells. Remaining recipient mice were infected with *N. brasiliensis*, and MLN cells were analyzed for CD44 and EGFP expression on gated CD4$^+$ T cells 10 days later.

ods for the detection of cytokine-producing cells were overcome with this system. Conventional methods for identifying cytokine-producing cells require the restimulation of cells ex vivo in order to allow detection of effector function (Openshaw et al., 1995; Richter et al., 1999). This typically involves activation using nonspecific stimuli in order to overcome the need to identify relevant antigens presented in vivo. Further, some methods require intracellular trapping of cytokine in the endoplasmic reticulum and cell permeabilization and abrogate the ability to study these cells further (Openshaw et al., 1995). As shown here, the 4get cells are substantially more sensitive for identifying IL-4-expressing cells than currently standardized methods. Comparison of the mean fluorescence intensity of cells from heterozygous and homozygous 4get mice demonstrated the capacity to quantitate 2-fold differences in expression, suggesting that expression can be detected from individual alleles using these cells. Under the conditions used—optimal concentrations of anti-CD3 and anti-CD28 antibodies—the percentages of EGFP$^+$ cells were comparable using cells from either heterozygous or homozygous 4get mice (Figure 2A; Table 1). These data suggest that monoallelic expression of the IL-4 gene occurs infrequently under these in vitro conditions, in contrast to the ease with which monoallelic expression of IL-4 has been documented using different systems (Bix and Locksley, 1998a; Riviere et al., 1998; Hu-Li et al., 2001). Although further studies are needed, initial analysis suggests that naive CD8$^+$ T cells, in contrast to naive CD4$^+$ T cells, may be incapable of expressing IL-4 from both alleles, at least under these conditions (Figure 2B).

After helminth infection, cells from 4get mice were activated in situ within relevant organs and required no further priming or stimulation for their identification. Unexpectedly, the first CD4$^+$ T cells to express EGFP in the lung on day 3, however, had a DX5^{lo} CD3^{lo} phenotype consistent with NK T cells (Bendelac et al., 1997). Prior studies in β2-microglobulin-deficient mice suggested that CD1-restricted NK T cells were not required for host immunity against *Nippostrongylus* (Brown et al., 1996). Indeed, in experiments not shown, these EGFP$^+$ cells did not stain with CD1d-α-GalCer tetramers that bind canonical Vα14/Vβ8.2 NK T cells (M.M., unpublished data; Benlagha et al., 2000; Matsuda et al., 2000). The localization to tissue, the rapid development of effector function, and the abrupt disappearance and slow repopulation following activation (Figures 4B and 4C) were observed after nonspecific stimulation of canonical NK T cells with α-GalCer, anti-CD3, or IL-12 (Eberl and MacDonald, 1998; Matsuda et al., 2000). Further study will be required to define fully both the lineage of these cells and the ligands that activate IL-4 gene expression after *Nippostrongylus* infection.

After day 4, a second population of CD4$^+$ T cells appeared in the lung and eventually became the dominant tissue effector cells. These cells were CD3^{hi} and displayed heterogeneous expression of DX5. We believe these cells are conventional CD4$^+$ T cells for several reasons. First, the kinetics of their appearance is consistent with the inherent delay reflecting their instruction from dendritic cells that must traffic from tissue sites of activation over 1–2 days during maturation from an antigen-capturing to an antigen-presenting phenotype (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2000). Indeed, in the mesenteric lymph node, CD4$^+$ T cells began to express the EGFP reporter above background levels on day 8, approximately 2 days after

the arrival of worms in the intestine on day 6 (Figure 4C). Second, infection with several virus models demonstrated the appearance of both $CD8^+$ and $CD4^+$ T cells that expressed a variety of markers typically associated with NK or NK T cells. Such markers included DX5, NK1.1, and Ly49 family members (Slifka et al., 2000; Kambayashi et al., 2001). Staining with virus peptide-specific MHC tetramers confirmed that these cells were conventional T cells. Indeed, we have demonstrated the appearance of the DX5 antigen on naive $CD4^+$ T cells 5 days after in vitro priming under either Th1 or Th2 conditions (M.M., unpublished data). The role of these induced NK-like markers on T cells is unclear, but activating receptors might reduce threshold signals required for the expression of TCR-dependent effector function in tissues (Groh et al., 2001).

The amounts of IL-4 produced by naive $\alpha\beta$ T cells—estimated to be 50 pg/10^6 cells in the initial 48 hr after activation—remain inefficient at Th2 priming in vitro (Noben-Trauth et al., 2000). The data here suggest that terminal Th2 differentiation and/or recruitment may occur in the tissues in response to IL-4 produced by resident or recruited NK T cells activated by migrating parasites. Prior studies in Stat6-deficient mice infected with *N. brasiliensis* demonstrated essentially wild-type levels of IL-4 and IL-13 in serum despite the inability to expel intestinal worms (Finkelman et al., 2000). As shown here, Stat6-deficient T cells efficiently activated EGFP expression in mesenteric lymph nodes but were unable to sustain stable IL-4 expression in tissues (Figure 5). Thus, these mice demonstrate that cytokine responses can be anatomically compartmentalized, possibly explaining the failure of worm expulsion in Stat6-deficient mice. Recent data suggest that initial activation of the IL-4 gene can be Stat6-independent, although subsequent stabilization required Stat6-mediated expression of GATA3 (Grogan et al., 2001); the latter transcription factor is necessary and sufficient for Th2 cell differentiation (Zheng and Flavell, 1997). Together with the data presented here, we favor a model whereby naive T cells become activated to express cytokines by Stat6-independent pathways in lymph nodes but become instructed to canonical, committed Th2 subsets in response to cytokines elaborated by resident specialized cell populations within inflammatory foci. These latter cells are prone to activation-induced cell death, as demonstrated for NK T cells, and cannot sustain tissue responses alone. Recent studies demonstrated a requirement for tissue Stat6 in the induction of chemokines required for Th2 cell recruitment to the lung (Mathew et al., 2001). Thus, in the absence of Stat6, IL-4/IL-13 expression cannot be sustained at tissue sites where these cytokines are required to mediate worm expulsion (McKenzie et al., 1999). These mice illustrate the value of the reporter in evaluating tissue-specific cytokine expression in vivo.

The capacity to recover effector cells from tissues should provide important reagents for homeostasis and memory studies, not only of canonical T cells, but also the NK T cells or other effector cells that express IL-4. Current methods often rely on the adoptive transfer of antigen-specific TCR transgenic T cells, which requires knowledge of relevant antigens prior to the analysis (Reinhardt et al., 2001). As shown here, transfer of $EGFP^+$ T cells to immunodeficient mice was followed by a loss of fluorescence as cells resided for 30 days in recipient mice. Although we have not thoroughly evaluated the half-life of EGFP in these cells, a half-life around 24 hr has been quantitated in mammalian cells (Li et al., 1998). The disappearance of EGFP that appears early after activation of cells under Th1 conditions (Grogan et al., 2001) and during homeostatic repopulation of TCR-Cα-deficient mice, as shown here (Figure 6), demonstrates that the protein does not persist indefinitely. The transferred cells were capable of reexpressing EGFP after infection, of conferring host protection, and of providing cytokine signals necessary for IgE production. This latter capacity likely reflects the preservation of a functional IL-4 gene in 4get mice, in contrast to prior IL-4 knockin models that resulted in deletion (Riviere et al., 1998; Hu-Li et al., 2001) or modification (Ho et al., 1998) of the endogenous gene. $CD4^+$ effector T cells as defined by EGFP expression were fully capable of conferring immunoprotection, extending to $CD4^+$ T cells similar observations demonstrating that $CD8^+$ T effector cells give rise to $CD8^+$ memory T cells (Jacob and Baltimore, 1999; Opferman et al., 1999). As such, T cell lines generated from these effector cells might prove useful for identifying protective worm antigens using various expression systems.

The ability to mark the IL-4 gene should be readily applicable to other cytokine loci, and the simultaneous use of different fluorescent markers for multiple genes should allow an unprecedented look at the evolving immune response in the whole animal. Together with the capacity to obtain these effector cells in high purity for further studies, such engineered mice will prove valuable reagents in models of immunoprotection, immunopathology, and autoimmunity.

Experimental Procedures

Mice

BALB/c mice (Jackson Laboratories, Bar Harbor, Maine), TCR Cα-deficient mice (Philpott et al., 1992), and Stat6-deficient mice (Kaplan et al., 1996) were kept under specific pathogen-free conditions in the animal care facility at the University of California, San Francisco.

Generation of IL-4-Reporter Mice

A 6 kb EcoRI fragment derived from 129/SvJ genomic IL-4 DNA was cloned into pgkTK (Tybulewicz et al., 1991). A SrfI site was introduced into the 3′ untranslated region upstream of the endogenous polyadenylation signal in exon 4 by site-directed mutagenesis using the following oligonucleotides: IL-4 Srf sense 5′-CGTAGTACT GAGCCCGGGCCATGCTTTAAC-3′ and IL-4 Srf antisense 5′-GTT AAAGCATGGCCCGGGCTCAGTACTACG-3′. The reporter cassette was derived from pIRES-EGFP (Clontech, Palo Alto, California). A Kozak translation initiation sequence (Kozak, 1992) was introduced upstream of the 11th ATG of the EMCV IRES element (Jackson et al., 1990) by PCR-mediated mutagenesis with the following oligonucleotides: IRES-3′ Nco 5′-CCATGGTATCATCGTGTTTTTCAAAGG-3′ and IRES-5′ 5′-CTGCAGGTCGAGCATGCATCTAGGG-3′. The modified IRES-EGFP cassette, including the bovine growth hormone polyadenylation signal, was ligated to the loxP-flanked *neo* cassette, *pL2neo2* (Gu et al., 1993), to generate the reporter/selection cassette. This cassette was cloned into the SrfI site of the modified genomic DNA to generate the final targeting construct.

PrmCre ES cells, which express the Cre recombinase under the protamine promoter (O'Gorman et al., 1997), were electroporated with the NotI-linearized targeting construct and selected in the pres-

ence of 400 μg/ml G418 and 2 μM gancyclovir. Resistant ES cell clones were screened for homologous integration by Southern blot. Targeted clones were injected into C57BL/6 blastocysts to create chimeric mice. The *neomycin*-resistance cassette was deleted in the male germline by Cre-mediated recombination after breeding chimeric mice to wild-type BALB/c females. Heterozygous animals were bred to BALB/c mice, and offspring were screened and selected for the presence of the reporter and the absence of the Cre transgene. Finally, heterozygous animals were interbred to obtain homozygous mice.

Cell Purification

Naive $CD4^+$ T cells were sorted from lymph nodes and spleen after labeling with anti-CD4-PE (YTS191.1, Caltag Laboratories, San Francisco, California) and anti-CD62L-APC (MEL-14, PharMingen, San Diego, California) using flow cytometry (MoFlo Multi-Laser Flow Cytometer, Cytomation, Ft. Collins, Colorado). Sorted cells were small, EGFP-negative lymphocytes on forward-side scatter analysis and were >99% $CD4^+$ $CD62L^{hi}$ in independent analysis. Naive $CD8^+$ T cells were sorted for the same parameters with anti-CD8-TriColor (Caltag Laboratories). Where designated, naive T cells were preincubated with the vital fluorescent dye, PKH26 (2 μM; Sigma Chemical Co., St. Louis, Missouri), as described (Zhang et al., 1997). Where noted, enriched $CD4^+$ T cells were prepared from spleen cells by the complement-mediated lysis of $CD8^+$ T cells, as described (Bix et al., 1998b).

Antigen-presenting cells (APC) were prepared from the spleens of TCR-Cα-deficient mice after the lysis of red cells and γ-irradiation (2500 rad), as described (Fowell et al., 1999).

In Vitro T Cell Priming

Purified naive $CD4^+$ or $CD8^+$ T cells in media (RPMI 1640 with 10% heat-inactivated fetal calf serum, 50 μM β2-mercaptoethanol, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin) were stimulated in 24-well plates at 10^6 cells/ml with mAbs to TCRβ (H57.597; 1 μg/ml) and CD28 (37N51.1; 5 μg/ml) and 50 U/ml recombinant human IL-2 (neutral priming) in the presence of 5×10^6 irradiated APC. Under Th1 priming conditions, murine recombinant IL-12 (5 ng/ml) and 20 μg/ml anti-IL-4 mAb (11B11), and, under Th2 priming conditions, murine recombinant IL-4 (50 ng/ml) and 50 μg/ml anti-IFN-γ mAb (XMG1.2), were added to the respective cultures. Where indicated, cells were washed after 5 days and restimulated with anti-TCRβ mAb and fresh irradiated APC. Supernatants were collected after 2 days and analyzed for cytokines by ELISA (Fowell et al., 1999).

Parasites and Infection

Third-stage larvae (L3) of *N. brasiliensis* were recovered from cultured feces of infected rats and washed extensively, and 750 organisms were injected subcutaneously in 0.2 ml PBS at the base of the tail, as described (Fowell et al., 1999). Mice were killed at designated times, and the presence of adult worms in the intestines was assessed by inverted microscopy. The whole lungs, spleens, mesenteric and peripheral (axillary and inguinal) lymph nodes were excised, minced, and dispersed into single-cell suspensions. Where designated, lung suspensions were purified further by centrifugation over Ficoll. Serum was collected for the determination of total serum IgE by ELISA, as described (Brown et al., 1996).

Flow Cytometric Analysis

Designated single-cell populations isolated from tissues, lymphoid organs, or in vitro cultures were labeled with the indicated conjugated mAb to cell surface markers (PharMingen and Santa Cruz Biotechnologies, Santa Cruz, California). Lung and spleen cells were preincubated with mAb to CD16/CD32 (Fc block; PharMingen) to block nonspecific binding. Analysis was acquired on a FACScalibur flow cytometer with CellQuest software (Becton Dickinson, Mountain View, California) after parameters were set by the use of isotype-matched control mAb and nonfluorescent cells from wild-type mice. Autofluorescence was readily distinguished from EGFP as assessed with cells from the latter mice.

Cytokine Assays

Mouse IL-4 and IFN-γ were determined by sandwich ELISA with detection limits of 50 pg/ml for IL-4 and 1 ng/ml for IFN-γ, as described (Fowell et al., 1999). ELISPOT assays were performed after priming T cells for 5 days, purifying cells over Ficoll, and restimulating with plate-bound anti-CD3 mAb for 6 hr as described (Brown et al., 1996).

Acknowledgments

The authors thank C. McArthur, N. Flores, and L. Stowring for technical assistance; N. Killeen and J. Dietrich for plasmids and blastocyst injections; B. Seymour and R. Coffman for parasites; M. Kronenberg for αGalCer-CD1 tetramers; and L. Lanier for constructive comments. This work was supported by Howard Hughes Medical Institute and AI30663 and HL56385 from the National Institutes of Health.

Received March 29, 2001; revised July 11, 2001.

References

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245–252.

Bendelac, A., Rivera, M.N., Park, S.-H., and Roark, J.H. (1997). Mouse CD1-specific NK1 T cells: development, specificity and function. Annu. Rev. Immunol. *15*, 535–562.

Benlagha, K., Weiss, A., Beavis, A., Teyton, L., and Bendelac, A. (2000). In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. J. Exp. Med. *191*, 1895–1903.

Bix, M., and Locksley, R.M. (1998a). Independent and epigenetic regulation of the interleukin-4 alleles in CD4+ T cells. Science *281*, 1352–1354.

Bix, M., Wang, Z.-E., Thiel, B., Schork, N.J., and Locksley, R.M. (1998b). Genetic regulation of commitment to interleukin 4 production by a CD4+ T cell-intrinsic mechanism. J. Exp. Med. *188*, 2289–2299.

Brown, D.R., Fowell, D.J., Corry, D.B., Wynn, T.A., Moskowitz, N.H., Cheever, A.W., Locksley, R.M., and Reiner, S.L. (1996). β2-microglobulin-dependent NK1.1 T cells are not essential for T helper cell 2 immune responses. J. Exp. Med. *184*, 1295–1304.

Eberl, G., and MacDonald, H.R. (1998). Rapid death and regeneration of NKT cells in anti-CD3ε- or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. Immunity *9*, 345–353.

Finkelman, F.D., Shea-Donohue, T., Goldhill, J., Sullivan, C.A., Morris, S.C., Madden, K.B., Gauser, W.C., and Urban, Jr., J.F. (1997). Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. Annu. Rev. Immunol. *15*, 505–533.

Finkelman, F.D., Morris, S.C., Orekhova, T., Mori, M., Donaldson, D., Reiner, S., Reilly, N.L., Schopf, N., and Urban, J.F., Jr. (2000). Stat6 regulation of in vivo IL-4 responses. J. Immunol. *164*, 2303–2310.

Fowell, D.J., Shinkai, K., Liao, X.C., Beebe, A.M., Coffman, R.L., Littman, D.R., and Locksley, R.M. (1999). Impaired NFATc translocation and failure of Th2 development in Itk-deficient CD4+ T cells. Immunity *11*, 399–409.

Grogan, J.L., Mohrs, M., Harmon, B., Lacy, D.A., Sedat, J.W., and Locksley, R.M. (2001). Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. Immunity *14*, 205–215.

Groh, V., Rhinehart, R., Randolph-Habecker, J., Topp, M.S., Riddell, S.R., and Spies, T. (2001). Costimulation of CD8αβ T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat. Immunol. *2*, 255–260.

Gu, H., Zou, Y.R., and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell *73*, 1155–1164.

Ho, I.-C., Kaplan, M.H., Jackson-Grusby, L., Glimcher, L.H., and

Grusby, M.J. (1998). Marking IL-4-producing cells by knock-in of the IL-4 gene. Int. Immunol. *11*, 243–247.

Hu-Li, J., Pannetier, C., Guo, L., Lohning, M., Gu, H., Watson, C., Assenmacher, M., Radbruch, A., and Paul, W.E. (2001). Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. Immunity *14*, 1–11.

Jackson, R.J., Howell, M.T., and Kaminski, A. (1990). The novel mechanism of initiation of picornavirus RNA translation. Trends in Biochem. Sci. *15*, 477–483.

Jacob, J., and Baltimore, D. (1999). Modelling T-cell memory by genetic marking of memory T cells in vivo. Nature *399*, 593–597.

Kambayashi, T., Assarsson, E., Chambers, B.J., and Ljunggren, H.-G. (2001). Expression of the DX5 antigen on CD8+ T cells is associated with activation and subsequent cell death or memory during influenza virus infection. Eur. J. Immunol. *31*, 1523–1530.

Kaplan, M.H., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity *4*, 313–319.

Kozak, M. (1992). Regulation of translation in eukaryotic systems. Annu. Rev. Cell Biol. *8*, 197–225.

Lanzavecchia, A., and Sallusto, F. (2000). Dynamics of T lymphocyte responses: intermediates, effectors and memory cells. Science *290*, 92–97.

Laouar, Y., and Crispe, I.N. (2000). Functional flexibility in T cells: independent regulation of CD4+ T cell proliferation and effector function in vivo. Immunity *13*, 291–301.

Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C.-C., and Kain, S.R. (1998). Generation of destabilized green fluorescent protein as a transcription reporter. J. Biol. Chem. *273*, 34970–34975.

Mathew, A., MacLean, J.A., DeHaan, E., Tager, A.M., Green, F.H.Y., and Luster, A.D. (2001). Signal transducer and activator of transcription 6 controls chemokine production and T helper type 2 cell trafficking in allergic pulmonary inflammation. J. Exp. Med. *193*, 1087–1096.

Matsuda, J.L., Naidenko, O.V., Gapin, L., Nakayama, T., Taniguchi, M., Wang, C.-R., Koezuka, Y., and Kronenberg, M. (2000). Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J. Exp. Med. *192*, 741–753.

McKenzie, G.J., Fallon, P.G., Emson, C.L., Grencis, R.K., and McKenzie, A.N.J. (1999). Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. J. Exp. Med. *189*, 1565–1572.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675–686.

Noben-Trauth, N., Hu-Li, J., and Paul, W.E. (2000). Conventional, naive CD4+ T cells provide an initial source of IL-4 during Th2 differentiation. J. Immunol. *165*, 3620–3625.

O'Gorman, S., Dagenais, N.A., Qian, M., and Marchuk, Y. (1997). Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. Proc. Natl. Acad. Sci. USA *94*, 14602–14607.

Openshaw, P., Murphy, E.E., Hosken, N.A., Maino, V., Davis, K., Murphy, K., and O'Garra, A. (1995). Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J. Exp. Med. *182*, 1357–1367.

Opferman, J.T., Ober, B.T., and Ashton-Rickardt, P.G. (1999). Linear differentiation of cytotoxic effectors into memory T lymphocytes. Science *283*, 1745–1748.

Ouyang, W., Lohning, G., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K.M. (2000). Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity *12*, 27–37.

Philpott, K.L., Viney, J.L., Kay, G., Rastan, S., Gardiner, E.M., Chae, S., Hayday, A.C., and Owen, M.J. (1992). Lymphoid development in mice congenitally lacking T cell receptor $\alpha\beta$-expressing cells. Science *256*, 1448–1452.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M.K. (2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101–105.

Richter, A., Lohning, M., and Radbruch, A. (1999). Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. J. Exp. Med. *190*, 1439–1450.

Riviere, I., Sunshine, M.J., and Littman, D.R. (1998). Regulation of IL-4 expression by activation of individual alleles. Immunity *9*, 217–228.

Slifka, M.K., Pagarigan, R.R., and Whitton, J.L. (2000). NK markers are expressed on a high percentage of virus-specific CD8+ and CD4+ T cells. J. Immunol. *164*, 2009–2015.

Tybulewicz, V.L., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C. (1991). Neonatal lethality and lymphopenia in mice with homozygous disruption of the c-abl proto-oncogene. Cell *65*, 1153–1163.

Urban, J.F., Jr., Noben-Trauth, N., Donaldson, D.D., Madden, K.B., Morris, S.C., Collins, M., and Finkelman, F.D. (1998). IL-13, IL-4Rα, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. Immunity *8*, 255–264.

Wang, F., Nemes, A., Mendelsohn, M., and Axel, R. (1998). Odorant receptors govern the formation of a precise topographic map. Cell *93*, 47–60.

Wills-Karp, M. (1999). Immunologic basis of antigen-induced airway hyperresponsiveness. Annu. Rev. Immunol. *17*, 255–281.

Zhang, X., Brunner, T., Carter, L., Dutton, R.W., Rogers, P., Bradley, L., Sato, T., Reed, J.C., Green, D., and Swain, S.L. (1997). Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid fas/fasL-mediated apoptosis. J. Exp. Med. *185*, 1837–1849.

Zheng, W.-P., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression. Cell *89*, 587–596.